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(54) Title: IMPROVEMENT OF AN EXPRESSION VECTOR FOR PRODUCTION OF RECOMBINANT PROTEINS**(57) Abstract**

The invention relates to a method of enhancing the transcription of a gene in a DNA construct incorporated into the genome of a eucaryotic host cell, which DNA construct comprises a structural gene for a desired protein or polypeptide and a gene promoter upstream of the structural gene. The invention resides in providing at least one enhancer element comprising the nucleotide sequence TTC TGA GAA upstream of said promoter, and exposing the DNA construct to lactogenic stimuli. The invention also relates to an expression vector, and a host cell and a transgenic mammal containing this vector.

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Improvement of an expression vector for production of recombinant proteins

Field of the invention.

The present invention relates to DNA plasmids to be used for the production of recombinant proteins. More specifically, the present invention concerns the addition of specific DNA elements to expression plasmids that serve a function as enhancing elements. The outcome is to improve the yields of recombinant protein production.

Background of the invention.

There are a number of different strategies for the large-scale production of recombinant proteins to be used in, for example, the pharmaceutical industry. In certain cases it is desirable that the recombinant protein is made in eucaryotic hosts. These hosts may be cultivated cells or animals made transgenic with respect to the gene of interest. In the latter situation, transgenic expression in milk is a valuable technique since transgenes, active in the mammary gland, have been described and milk is a readily available body fluid.

The present invention relates to, in an unrestricted way, an improvement in expression vectors used to produce recombinant proteins in milk. These improved expression vectors will increase the yield of valuable recombinant proteins which will be of value for the facilitation of subsequent handling and purification steps.

Construction of a transgene requires certain basic ingredients, one being the structural gene containing the coding information for the protein of interest. A basal eucaryotic gene expression promoter is also required. In addition, other sequences can be used that confer tissue specificity or enhance expression in response to stimulus. The present invention relates to a specific type of enhancers, namely enhancers responding to hormonal stimuli. The particular enhancer in question is a sequence of DNA that confers a response to signals

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evoked by pituitary hormones belonging to the group of lactogenic hormones such as prolactin (Prl) and placenta lactogen (PL) and somatogenic hormones such as growth hormone (GH). Both of these groups of hormones occupy central roles in the stimulation of mammary gland development and function. The present invention concerns the definition of enhancers responding to both lactogenic and somatogenic hormones and the construction of expression vectors, that, in their ability to respond to both lactogenic and somatogenic hormones, will function in an improved manner as transgenes for production of recombinant proteins in milk.

Previous studies have defined a gene, the Serine Protease Inhibitor 2.1 (SPI) gene, that responds to GH. In the 5' flank of this gene a DNA element has been identified that enhances gene expression in a GH-dependent fashion. The sequence of this GH response element (SPI GH-RE) in question is:

GATCTACGCTTCTACTAATCCATGTTCTGAGAAATCATC
CAGTCTGCCCCATG, (Yoon et al. J. Biol. Chem. 265; 19947 (1991))

Within this sequence we now disclose a shorter "SPI-GAS like element"; TTCTGAGAA, that constitutes the core GH regulated sequence. As exemplified below the SPI-GAS element is also functional when transferred to a reporter gene such as the Luciferase gene (Sliva D. et al J. Biol. Chem. in press). In the following we also disclose that the GH-regulated sequences described above are also regulated by prolactin and that this can be used to design new expression vectors that improve existing vectors used to produce recombinant proteins in milk.

Examples

Example 1. Identification of a core GH regulated sequence.

The 50 bp SPI-GHRE;
(GATCTACGCTTCTACTAATCCATGTTCTGAGAAATCATC
CAGTCTGCCCCATG) was used to identify a core GH regulated sequence using gel electrophoresis mobility shift assay (GEMSA). Nuclear extracts were prepared and incubated with a ³²P labelled 50 bp SPI-GHRE. Subsequently the extracts were analysed on polyacrylamide gels. The results showed that nuclear proteins, dependent on GH, bound to this DNA sequence. By competition with shorter oligonucleotides derived from SPI-GHRE a core GH sequence was identified. Based on certain sequence homologies to interferon response-elements we called this sequence SPI-GAS and also demonstrated that SPI-GAS functions as a GH regulated DNA

element when put into a reporter vector. The core SPI-GAS has the following sequence; TTCTGAGAA.

Example 2. Prolactin and growth hormone both activate SPI-TK-reporter gene.

An expression plasmid containing a recombinant hormone responsive reporter consisting of six repeats of a 50 bp growth hormone responsive element (GH-RE) from the serine protease inhibitor (SPI) 2.1 promoter fused to the thymidine kinase (TK) promoter was constructed. Corresponding constructs were made using the SPI-GAS element. Variants expressing either the bacterial protein chloramphenicol acetyl transferase (CAT) or firefly luciferase (SPI-CAT or SPI-Luc respectively) cDNAs were then constructed. Techniques to make these vectors are well known to experts in the field. The plasmid DNA constructions were transfected, together with plasmid expression vectors encoding either rat growth hormone receptors or mouse prolactin receptors, into Chinese hamster ovary (CHO), COS, and Buffalo rat liver (BRL) cells, using DOTAP liposomes and according to the manufacturer instructions. Cells were incubated overnight with DNA and DOTAP in serum free media, left and then exposed to growth hormone or prolactin for 12 hours. Cell lysates were then prepared and CAT or luciferase enzyme activity measured. Both growth hormone and prolactin treatment lead to an approximately 5-fold stimulation reporter enzyme expression relative to transfected but non-hormone treated cells. These results show that both growth hormone and prolactin can regulate the reporter construct and that a requisite for this is the presence of SPI elements. The core element in the SPI-TK-reporter gene that confers GH regulation is likely to be; TTCTGAGAA, and similar results can be obtained with this element termed SPI-GLE as with the longer, 50 bp element named SPI-GHRE.

Example 3. Multimeric SPI elements in front of a TK promoter give a better response.

Reporters plasmids containing one to six copies of the 50bp SPI element fused to the TK promoter were constructed. The growth hormone responsiveness of these constructs was tested by transfection into a CHO cell line that stably expresses the rat growth hormone receptor DNA. Growth hormone stimulation of these cells showed that multimerization of SPI elements resulted in a larger growth hormone response.

Example 4. Expression of stable incorporated SPI-TK- Luciferase is growth hormone regulated.

To demonstrate that SPI elements retain growth hormone responsiveness function when genomically integrated CHO cells were transfected with the three following plasmids: SPI-LUC (described in example 1), an expression vector containing the CMV promoter and rat growth hormone receptor cDNA and a neomycin expression vector. Neomycin resistant clones were tested for growth hormone response by exposing cells to growth hormone for 12 h under serum free conditions and then measuring luciferase activity in cell lysates. The results indicated a three-fold growth hormone-regulated induction of expression of the stably integrated reporter gene.

Example 5. SPI elements in front of a strong promoter (SV40) results in a protein production that is further enhanced by GH.

Six copies of the SPI element were introduced upstream of a strong CMV promoter driving expression of the CAT cDNA in a plasmid construct. This construct was transfected into CHO-4 cells and GH regulation was tested as described above. It was found that GH stimulated the production of CAT.

CLAIMS

1. A method of enhancing the transcription of a gene in a DNA construct incorporated into the genome of a eucaryotic host cell, said DNA construct comprising a structural gene for a desired protein or polypeptide and a gene promoter upstream of the structural gene, **characterized** by providing upstream of said promoter at least one enhancer element comprising the nucleotide sequence TTC TGA GAA, and exposing the DNA construct to lactogenic stimuli.
2. The method according to claim 1, **characterized** in that said enhancer element is the SPI-growth hormone responsive element (SPI-GHRE) or a derivative thereof.
3. Use of an enhancer element comprising the nucleotide sequence TTC TGA GAA in an expression vector to be used in a non-human mammal for the production of recombinant proteins or polypeptides in milk.
4. The use according to claim 3, wherein said enhancer element comprises a single or multimeric copies of the SPI-growth hormone responsive element (SPI-GHRE) or a derivative thereof.
5. An enhancer element which when used in a DNA construct for transfection of a eucaryotic host cell is responsive to hormonal stimuli, **characterized** in that said enhancer element comprises the nucleotide sequence TTC TGA GAA, with the proviso that said nucleotide sequence is not the DNA sequence of the SPI-growth hormone responsive element (SPI-GHRE).
6. The enhancer element of claim 5, **characterized** in that it is responsive to both somatic and lactogenic stimuli.

7. The enhancer element of claim 5 or 6, **characterized** in that it is responsive to signals generated from both growth hormone and prolactine receptors.
8. An expression vector comprising a structural gene encoding a desired protein or polypeptide and a promoter, **characterized** in that the vector further comprises at least one enhancer element including the nucleotide sequence TTC TGA GAA, with the exception of the SPI-growth hormone responsive element (SPI-GHRE).
9. An expression vector comprising a structural gene encoding a desired protein and a mammary tissue specific promoter, **characterized** in that it further comprises at least one enhancer element including the nucleotide sequence TTC TGA GAA.
10. The expression vector according to claim 9, **characterized** in that said enhancer element comprises a single or multimeric copies of the SPI-growth hormone responsive element (SPI-GHRE) or a derivative thereof.
11. A eucaryotic host cell containing the expression vector according to claim 8, 9 or 10.
12. A transgenic non-human mammal having incorporated into its genome a DNA construct comprising a structural gene encoding a desired protein or polypeptide linked to a control sequence for expression in milk-secreting epithelial cells of the mammary gland so that the protein or polypeptide is secreted into the milk, **characterized** in that said DNA construct further comprises at least one enhancer element which includes the nucleotide sequence TTC TGA GAA and is responsive to signals generated from prolactine receptors.

13. The transgenic non-human mammal according to claim 12, characterized in that it is selected from mouse, pig, goat, sheep and cow.

14. A method for producing a recombinant protein or polypeptide, characterized by providing a transgenic non-human mammal according to claim 12 or 13, and recovering the protein or polypeptide from the milk produced by the mammal.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/SE 95/01235

A. CLASSIFICATION OF SUBJECT MATTER

IPC6: C12N 15/85, A01K 67/027, C12N 5/10

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC6: C12N, A01K, C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EDOC, MEDLINEE, BIOSIS, DBA

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 9405796 A1 (AMERICAN RED CROSS), 17 March 1994 (17.03.94), page 6, line 1 - line 11, the claims --	1-14
X	EP 0420055 A2 (W.R. GRACE & CO.-CONN.), 3 April 1991 (03.04.91), the claims --	1-14
X	WO 8801648 A1 (IMMUNEX CORPORATION), 10 March 1988 (10.03.88), page 3, line 8 - line 13 --	1-14

☒ Further documents are listed in the continuation of Box C.
 ☒ See patent family annex.

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INTERNATIONAL SEARCH REPORT

International application No.

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C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p data-bbox="332 315 1193 525">Dialog Information Service, file 154, Medline, Dialog accession No. 06192046, Medline accession no. 87166046, Yoon JB et al: "Growth hormone induces two mRNA species of the serine protease inhibitor gene family in rat liver", J Biol Chem (UNITED STATES) Mar 25 1987, 262 (9) p 4284-9</p> <p data-bbox="673 556 812 609">-----</p>	1-14

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Information on patent family members

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Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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EP-A2- 0420055	03/04/91	AU-B- 649407	26/05/94
		AU-A- 6259590	28/03/91
		JP-A- 3210187	13/09/91
		US-A- 5320952	14/06/94
WO-A1- 8801648	10/03/88	AU-A- 7879987	24/03/88